

# CAG Repeat Lengths in X- and Y-bearing Sperm Indicate That Gender Bias during Transmission of Huntington's Disease Gene Is Determined in the Embryo\*

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**The size of the CAG tract at the Huntington's disease (HD) locus upon transmission depends on the gender of the parent. However, the basis for the parent-of-origin effect is unknown. To test whether expansion and contraction in HD are "imprinted" in the germ cells, we isolated the X- and Y-bearing sperm of HD transgenic mice. Here we show that CAG repeat distributions in the X- and Y-bearing spermatozoa of founding fathers do not differ. These data show that gender-dependent changes in CAG repeat length arise in the embryo.**

The mechanism for expansion in HD<sup>1</sup> and other trinucleotide disorders is not known. However, one of the poorly understood features of expansion is the gender bias that is associated with transmission. Large changes in repeat number are known to be transmitted through the paternal line in HD (1, 2), SCA1 (3), dentatorubral-pallidoluysian atrophy (DRPLA) (4), Machado-Joseph disease (5), and spinal and bulbar muscular atrophy (SBMA) (6) and through the maternal line in Fragile X (7, 8). The gender bias transmission has also been documented in transgenic mice (9–12). Similar to humans, HD transgenic mice transmit expansions predominantly through the male germ line (9, 10, 12). Moreover, we found that expansion and contraction of CAG repeat size in the *hHD* transgene depended on the gender of the progeny (9).

Expansions are primarily seen in males and contractions dominate in females. The molecular basis for the parent-of-origin effect is unknown. The gender dependence of expansion can be due to differences in CAG repeat distribution in X- and Y-bearing sperm. Alternatively, the change in repeat number, contraction, or expansion can take place after fertilization in early embryogenesis. In order to distinguish between these two mechanisms, the isolation and purification of the X- and Y-bearing sperm were required. This, however, was not possible using conventional technology.

In present work, we have developed techniques and used

advanced technology to separate pure populations of X- and Y-bearing germ cells from HD transgenic mice. We show here that CAG repeat distributions in X- and Y-bearing parental sperm are not different. Repeat expansion occurs equally well within both X and Y chromosomes. This indicates that the gender-dependent processing of CAG repeats must take place post-zygotically. Thus, these data provide evidence for a new kind of imprinting that may be important in the interpretation of genetic data in other systems.

## EXPERIMENTAL PROCEDURES

**Animals—HD transgenic male mice (line B6CBA-TgN R6/1)** were originally purchased from The Jackson Laboratory. The colony was maintained at the animal core facility, Mayo Clinic/Foundation. Animals were routinely screened for the presence of *HD* transgene by PCR (10, 18).

**Preparation of Single Cell Suspension and Flow Sorting—**Mouse epididymis was dissected from the animal, placed in phosphate-buffered saline, and chopped with a razor. Resulting suspension was filtered through cheesecloth. The supernatant was centrifuged at 1500 rpm, and the pellet was resuspended in phosphate-buffered saline. Mature spermatozoa then were sonicated, stained with Hoechst 33342 (5  $\mu$ g/ml), and subjected to FACS analysis. Sperm samples were sorted using a modified FACSVantage (BD Biosciences, San Jose, CA) with argon laser operating in the ultraviolet spectrum. The fluorescence emission was collected at 0 and 90° to the excitation source through 418-nm long pass filters.

**CAG Repeat Sizing—**Sorted X- and Y-bearing sperm were collected by centrifugation and lysed. DNA was isolated, and CAG repeat lengths were determined by PCR followed by GeneScan analysis as described in Refs. 10 and 18.

**FISH Analysis—**Aliquots of spermatozoa, sorted for X and Y chromosomes, were analyzed by double-FISH. The sperm cells were decondensed with 10 mM dithiothreitol, denatured using 70% formamide, and subsequently hybridized with specific probes for mouse X and Y chromosomes. The probes were centromere-specific  $\alpha$ -satellite DNA that was directly labeled with a fluorescent dye. The X chromosome was labeled with SpectrumOrange and the Y chromosome with SpectrumGreen (Imagenetics). The spermatozoa were visualized and classified as either X- or Y-bearing using a Nikon Optiphot-2 microscope equipped with dual pass fluorescein isothiocyanate/rhodamine filter.

## RESULTS

To test whether expansion and contraction in HD are "imprinted" in Y- and X-bearing germ cells, we adapted a FACS approach to separate X- and Y-bearing spermatozoa (Fig. 1, A–C), after which we sized the CAG repeat in each population (9, 10) (Fig. 1D). We found that X- and Y-bearing sperm did not differ in repeat distribution in any animal tested (Fig. 1D; shown are two animals).

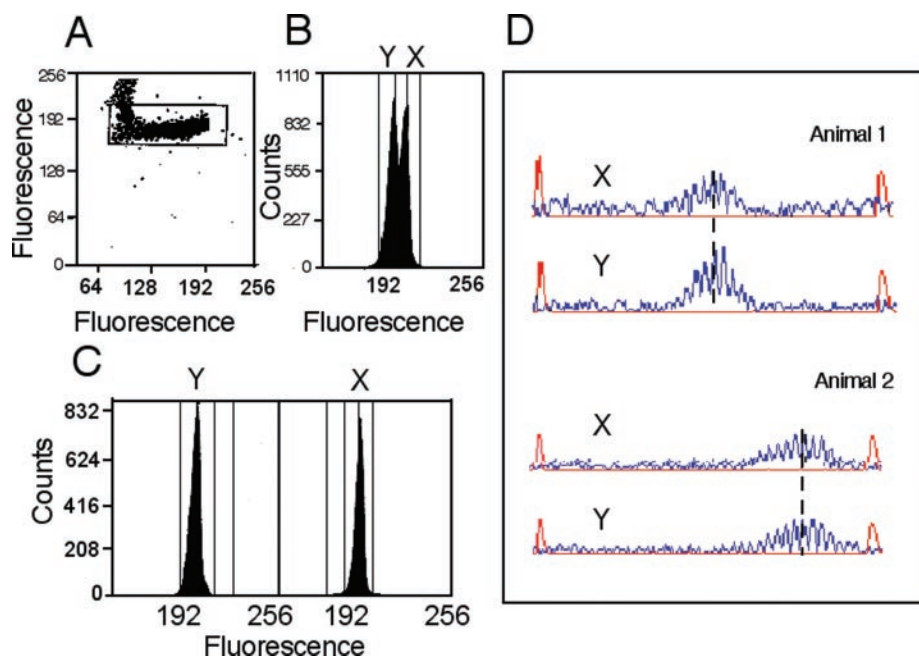
These results were not due to population mixing. We were able to isolate successfully pure populations of X and Y chro-

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<sup>1</sup> The abbreviations used are: HD, Huntington's disease; FACS, fluorescence-activated cell sorting; FISH, fluorescence *in situ* hybridization.

**FIG. 1. CAG repeat lengths do not differ in X- and Y-bearing spermatozoa.** A, profile of sorted cells. Highlighted, properly oriented spermatozoa (gated population) were detected by a 90° fluorescence detector. B, the fluorescence profile from a 0° detector is shown (gated population). The two peaks represent populations enriched in Y and X sperm (left and right peak, respectively). C, aliquots of sorted populations were reanalyzed. D, gene scan analysis of CAG repeat distribution in populations of sorted spermatozoa. The traces of the PCR products were obtained and analyzed using GeneScan Analysis version 3 (PerkinElmer Life Sciences). The peak with the largest area was taken as the midpoint of the peak distributions (shown as dashed line) in gene scan traces after normalization with respect to internal standards (shown in red). Data for two individual animals are shown.



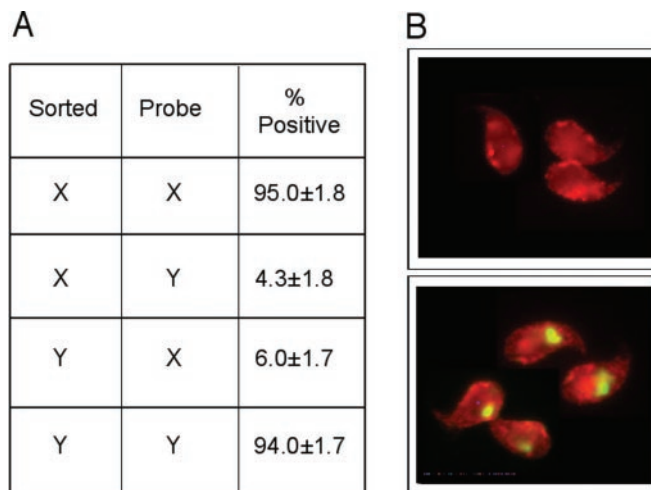
mosome-bearing spermatozoa based on the 2.8–3% total DNA content differences. Isolations were performed using a flow cytometer with specific modifications as developed by Johnson *et al.* (13). Modifications included an additional fluorescence detector positioned at a right angle (90°) and a novel sample injection tip designed to “orient” the spermatozoa to the laser beam (14, 15). Fluorescent signal from Hoechst-stained spermatozoa oriented with their brightest edge toward the 90° detector was collected by 0° detector and showed bimodal distribution. The smaller left peak comprised Y-bearing sperm and the larger right peak comprised X-bearing sperm (Fig. 1, A–C). The two peaks overlap (Fig. 1B). Non-overlapping areas of the peaks representing populations enriched in Y and X sperm (left and right peak, respectively) were selected by the electronic sort windows and collected (Fig. 1C).

The purity and accuracy of the isolated X- and Y-bearing sperm were confirmed by FISH analysis using specific X and Y probes (Fig. 2, A and B). Mean percentages of X- and Y-bearing spermatozoa indicate 94–95% efficiency of separation in tested samples (Fig. 2A). Nonparametric Wilcoxon Mann-Whitney test (9) was used to compare repeat distributions (Fig. 1D) of two sorted populations. Analysis confirmed that neither the size of the midpoint (peak with the largest area) nor the distribution of the repeat sizes was different between X- and Y-bearing spermatozoa.

#### DISCUSSION

It has been well documented that in both HD patients (16, 17) and transgenic mice (18) expansions are present in spermatozoa. Repeats after transmission tend to expand in male progeny in mice (9), and new mutations tend to arise through the paternal line in humans (19). Thus, expansion is gender-biased. Direct testing of the gender dependence, however, has not been possible by using conventional technology. Questions were raised as to whether altered repeat sizes existed on the X and Y chromosomes of the parent before transmission. In this work, we directly tested whether expansions and contractions are segregated with X- and Y-bearing spermatozoa. We demonstrate here that the CAG repeat distributions in X- and Y-bearing parental sperm are not different (Fig. 1D). The repeat expansion occurs equally well within both X and Y chromosomes.

GeneScan is accurate in showing the most prevalent sizes in



**FIG. 2. Double-FISH analysis of the sorted spermatozoa.** A, sorted spermatozoa stained with specific probes for mouse X and Y chromosomes. The X chromosome was labeled with SpectrumOrange and the Y chromosome with SpectrumGreen. B, summary of quantification verifying accuracy of flow cytometry. The spermatozoa were visualized and classified as either X- or Y-bearing using a Nikon Optiphot-2 microscope equipped with dual pass fluorescein isothiocyanate/rhodamine filter.

the heterogeneous pool (9, 10, 12, 18). Therefore, the data are clear in evaluating repeat length alterations. We have reported previously (9) rigorous statistical analysis using GeneScan data to establish the significance of the gender bias in whole animal populations. Although single cell analysis (16, 17) and small pool PCR (20–22) have been used to size repeat lengths in human studies, the advantage to these methods is the ability to detect rare changes in the repeat sizes. We find that gene scan analysis carried out on the total population of germ cells in mice is accurate in distinguishing changes between X and Y populations of sperm.

These data for the first time allow an unequivocal demonstration that the observed gender bias was not due to imprinting of the parental sperm. Rather, the gender-dependent processing of CAG repeats within the X and Y chromosomes appears to occur post-zygotically. These data point to a new kind of imprinting that depends on signals in the embryo and

may influence far-reaching implications with respect to the interpretation of genetic data.

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